ARACHIDONIC ACID ACCUMULATION AND Δ5-DESATURATION IN FELINES AFTER FEEDING A ɣ-LINOLENIC ACID ENRICHED DIET

A Thesis

by

AMY JO CHAMBERLIN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2009

Major Subject: Nutrition

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Approved by:

Chair of Committee,	John E. Bauer
Committee Members,	Stephen B. Smith
	Guoyao Wu
Intercollegiate Faculty Chair,	Stephen B. Smith

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ABSTRACT

Arachidonic Acid Accumulation and Δ5-desaturation in Felines After Feeding a ɣ-linolenic Acid Enriched Diet. (December 2009)

Amy Jo Chamberlin, B.S., University of Nebraska - Lincoln

Chair of Advisory Committee: Dr. John E. Bauer

Feline lipid metabolism is a topic for greater exploration due to this specie's unique characteristics. Cats express limited Δ6-desaturase activity necessary for conversion of linoleic acid (LA, 18:2n-6) to arachidonic acid (AA, 20:4n-6). The possibility exists that ɣ-linolenic acid (GLA, 18:3n-6) may serve as a precursor of AA in reproductive tissues especially if coupled with chain elongation and a functionally active Δ5-desaturase. In addition no research has been conducted regarding feline reproductive Δ8-desaturase activity as an alternate to the production of AA.

To investigate desaturation activities, a group of 26 adult female cats were randomly assigned into 1 of 3 groups based on the diet fed: High Linoleic Acid (HL, n=7), Low Linoleic Acid (LL, n=9), and High ɣ-Linolenic Acid (GLA, n=10).The diets were fed for 300 days prior to ovariohysterectomy at which time EDTA plasma and ovarian, uterine, and subcutaneous adipose tissues were collected. Homogenates of each tissue were prepared and frozen in aliquots at -80°C. Total lipids were extracted from the plasma and tissue homogenates followed by phospholipid (PL) fractionation via thin layer chromatography and fatty acid (FA) analyses by gas chromatography. The Shapiro-Wilks

test was used to determine normal distribution of FA data followed by One-Way ANOVA and Tukey multiple comparisons ($p<0.05$). Plasma PLs were significantly increased in both GLA and dihomo-ɣ-linolenic acid (DGLA, 20:3n-6Δ8,11,14) in the GLA group and statistically increased in 20:2n-6 and 20:3n-6(Δ5,11,14) in the HL group. Uterine tissue homogenates had significantly increased amounts of DGLA and AA, however ovarian tissue showed an increase of only DGLA. Adipose tissue FAs showed significantly high amounts of DGLA in the GLA group.

It is concluded that a high GLA diet results in increased AA in uterine, but not ovarian, tissues and thus may supply eicosanoid precursors in support of reproduction. The presence of increased amounts of 20:3n-6(Δ5,11,14) and not AA in the plasma and uterine tissues in the HL group suggests that Δ6-desaturase cannot be induced and that Δ8-desaturase is not active when feeding high dietary LA. Furthermore, the increase in DGLA may provide an adipose storage reservoir for additional conversion under times of metabolic need. These data support the presence of a functionally active Δ5-desaturase in uterine, but not ovarian, tissues. The findings also suggest that increased dietary GLA may be used to meet the AA requirements for reproduction in cats in the absence of an animal based pre-formed source of AA.

DEDICATION

With all my heart, I would love to dedicate this thesis to my favorite Uncle Larry. Aside from my parents, he was always my biggest fan. Whether it was supporting me in athletics or making sure the boys weren't giving me trouble, he was always there with something to say. I will always love and miss you every day.

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Next I would like to thank my parents, Roger and Pam, and my brother, Dave. I by far have the most caring, helpful, and amazing family anyone could ask for. I cannot count the number of times my dad and brother have packed up U-Hauls and drove thousands of miles just because I decided to move…again! Not many people are as fortunate as I am to have such a wonderful family that not only believes in you, but would do anything for you at the drop of a dime. I truly appreciate all that you three have done for me over the years and will never be able to thank you enough. I thank God every day that you all are in my life! I love you guys so much and look forward to being back closer again!

I would next like to thank my friends. I am incredibly fortunate to have such an amazing group of friends not only in Nebraska, but in Texas now as well. Without the support of my girls back home I would not be where I am today. I truly love all of you

and look forward to returning and starting a new chapter of my life with y'all! As for the crazy group of friends I have acquired in Texas, I couldn't thank you enough as every one of you helped make my transition to Texas wonderful. Without all of you, this experience would not have been worth it and I truly love and will miss you every day!

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NOMENCLATURE

AA	Arachidonic Acid
AAFCO	Association of American Feed Control Officials
ALA	Alpha-Linolenic Acid
BCA	Bininchoninic Acid
BSA	Bovine Serum Albumin
CL	Cardiolipin
CPM	Counts Per Minute
DGLA	Dihomo-gamma Linolenic Acid
EFA	Essential Fatty Acid
FA	Fatty Acid
FAME	Fatty Acid Methyl Ester
HPTLC	High Performance Thin Layer Chromatography
GC	Gas Chromatography
GLA	Gamma-Linoleic Acid
HUFA	Highly Unsaturated Fatty Acid
LARR	Laboratory Animal Research and Resources
LPC	Lyso-phosphatidylcholine
MCT	Medium Chain Triglycerides
MUFA	Monounsaturated Fatty Acid
NRC	National Research Council

PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PL	Phospholipid
PS	Phosphatidylserine
PUFA	Polyunsaturated Fatty Acid
SFA	Saturated Fatty Acid
SPH	Sphingomyelin
TLC	Thin Layer Chromatography
TSH	Thyroid Stimulating Hormone

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CHAPTER I

INTRODUCTION

Mammalian Fatty Acid Metabolism. Fatty acid metabolism is an essential process in living organisms. The synthesis and degradation of FA allows for both energy storage and proper organism function. Fatty acid biosynthesis varies amongst species, therefore not all animals possess the same requirements [1]. Mammals, including cats, and most ruminants, possess the ability to synthesize both nonessential saturated and monounsaturated fatty acids (MUFA) *de novo* from amino acids or glucose via acetyl CoA [2]. Concerns arise in mammalian species regarding their inability to synthesize certain polyunsaturated fatty acids (PUFA) and their highly unsaturated derivatives (HUFA), from acetyl CoA due to lack of specific enzyme activities. However, mammals can synthesize HUFAs when the diet is supplied with PUFA precursors, LA and α-linolenic acid (ALA, 18:3n-3) [3]. Thus, both LA and ALA are classified as dietary essential fatty acids (EFA), because they cannot be synthesized and because they functionally contribute in important ways to benefit animal health [2,4].

A deficiency in the EFA, LA, can lead to dramatic and harmful effects on the well-being of an animal. Such cases include improper cellular development and reproduction, impaired growth and renal functions, skin lesions, and other problems [5].

This thesis follows the style of Lipids.

In rats, it was discovered that LA was more effective than ALA at curing the common clinical signs of EFA deficiency, including reduced growth rate and hair loss. EFA requirements can be species-specific especially where AA is concerned. Because little to no AA is found in plant sources, obligate carnivorous species, such as cats, have an essential requirement for this FA compared with other mammals. Conversely, dogs and most other mammals possess the ability to desaturate EFAs, synthesize AA, and do not have an essential requirement for it in comparison to obligate carnivores [6]. For example, Dunbar et al. [6] found, using ^{14}C radio-labeled LA, that dog liver microsomes possess the ability to desaturate EFAs. This study also utilized rat liver microsomes as a positive control group for the Δ6 enzymatic activity determinations because rats were known to possess an active Δ6-desaturase enzyme [6-8]. Indeed, most previous studies have used the rat species as a key model for investigating PUFA metabolism due to a highly active Δ6-desaturase enzyme. Because of differences in EFA requirements among species it is useful to investigate comparative aspects of species-specific FA metabolism to provide a better understanding of their similarities and differences.

Unique Fatty Acid Metabolism in Cats. The domestic feline is a unique species with respect to FA metabolism. This obligate carnivore possesses specialized requirements for dietary animal fat and other nutrients to meet its nutritional needs. As previously mentioned, cats, like other mammals, are incapable of synthesizing LA making it an EFA. In contrast to most mammalian species, however, cats express limited capacity to synthesize AA from LA [9]. Consequently, AA is a FA that is conditionally essential for both kittens and adult queen reproduction [5]. In feline reproduction, AA is

required for several reasons including higher conception rates, viable litters, proper kitten growth, successful reproduction, decreased congenital defects, and increased litter size [2,4-5]. When reproducing queens were fed diets low in polyunsaturates, including LA, ineffective reproduction occurred, however normal reproductive functions were restored with the addition of AA into the diet [5]. When AA was completely devoid from the diet of reproducing queens, negative outcomes resulted. Although queens were capable of entering estrus, mating, and gaining subsequent weight displaying normal pregnancy conditions, after delivery, most of the live kittens were cannibalized [2,5].

Although AA is essential for kitten growth and queen reproduction, male cats may be able to synthesize sufficient AA for this purpose. When male cats were fed an AA deficient diet containing LA and bred with an AA sufficient queen, viable, clinically normal litters were produced [5]. In view of these results, it is evident that male cats do not require AA for reproductive capacity. However, according to MacDonald et al. [10], dietary AA may be essential in male cats for reasons other than reproduction as the AA synthesized in the testes was not released into the blood stream. These data along with the importance of AA in queens raises questions as to how much AA is truly necessary for felines to maintain these functions. The recommended allowance for adult cat maintenance according to The Nutrient Requirements for Dogs and Cats (2006) [11] is 0.025g/1,000 kcal metabolizable energy. MacDonald et al. [10] showed that queens fed AA at 0.04% of energy displayed essentially normal reproductive behaviors. Such a diminutive requirement might possibly suggest AA conversion via alternate pathways or

perhaps recycling occurring in various tissues. Nonetheless, few studies exist that demonstrate either of these 2 possibilities.

Because AA is a conditionally essential FA for reproduction of queens and for kitten growth, investigating dietary modalities for increasing AA production as well as adequate dietary additive amounts in queens is a valuable research topic in feline nutrition. The pathway of AA synthesis from LA begins with a rate limiting Δ6-desaturation step, followed by chain elongation, and finally Δ5-desaturation [12]. This sequence of reactions has been demonstrated in several studies to be limited in cats due to the deficiency of a fully functional Δ6-desaturase enzyme. Evidence for this phenomenon was first demonstrated by Rivers et al. [13] who observed that feline liver preparations lacked the Δ6-desaturase enzyme required for the conversion of LA to AA. Rivers et al. [13] concluded that cats are incapable of chain elongating or desaturating LA as only small amounts of AA and Δ11,14-eicosadienoic acid (20:2n-6) were observed in spite of a very high amount of LA present in the tissue lipids and diet. Consequently when Δ6-desaturase activity is limited or low a dietary requirement for AA is necessary [7].

Dietary Induction of Δ6-Desaturase. As noted, AA synthesis from LA involves the rate limiting Δ6-desaturation step. When a species, such as the cat, lacks an essential component for AA synthesis, providing dietary amounts of other FA precursors may support alternate pathways. Discovery of limited Δ6-desaturase in cats was a novel finding at the time of Rivers et al. [13] because it was assumed all species had a similar high rate of Δ6-desaturation. Sinclair et al. [7] provided early evidence that when cats

were fed a diet consisting mainly of saturated fat plus LA, an increase of both 20:3n-6(Δ5,11,14) and 20:2n-6 was observed. His data suggested that LA is not converted to AA to any extent via the normal pathway in liver tissues of cats. They then speculated that LA was instead, chain elongated to 20:2n-6, which could serve as a substrate for Δ5-desaturation [12]. However the total dietary amount of LA in Sinclair et al. [7] studies was not reported therefore, the possibility exists that some potentially higher amount of LA may induce the limiting Δ6-desaturase activity. One objective of the present study was to investigate whether Δ6-desaturase may be induced when cats were fed dramatically high amounts of LA.

Evidence for Δ5-Desaturation. Even though cats do not possess sufficient Δ6-desaturase for conversion, some studies have found that this species does, in fact, possess a small amount of Δ5-desaturase [4,12]. When diets rich in LA (hydrogenated beef fat plus safflower seed oil in the ratio 4.3:1) were fed to cats, a modest accumulation of 20:2n-6 appeared suggesting an alternative chain elongation step as well as the emergence of a novel FA 20:3n-6(Δ5,11,14) which may be the result of Δ5-desaturation [9,14]. Sinclair et al. [14] reported 2 studies which suggested the presence of an active Δ5-desaturase in cats. This group orally dosed ^{14}C radio-labeled DGLA into felines. The cats were euthanized 22 hours later at which time liver tissue was extracted and analyzed resulting in increased AA radioactivity [14]. They also demonstrated that when ^{14}C radio-labeled GLA was fed, both AA and DGLA increased in feline erythrocyte lipids [9,14-15]. When GLA was added to diets of queens during reproduction, AA status improved suggesting the Δ6-desaturase step was likely by-passed [9]. The accumulation

of these FAs demonstrated the possibility of an active Δ5-desaturase enzyme in the feline species [7].

Similar work has been conducted in other species including the rat. Brenner et al. [16] estimated Δ5 and Δ6-desaturase in hepatic microsomes using ^{14}C radio-labeled DGLA and found conversion to AA through an active Δ5-desaturase. His enzymatic activity data of 1.165±0.201 expressed as nm DGLA product per minute times mg protein, demonstrated relatively high percent conversion of DGLA to AA in the rat liver. Earlier, Blond et al. [17] isolated rat liver microsomes and, using exogenous ^{14}C radio-labeled DGLA, showed conversion to AA demonstrating an active Δ5-desaturase in the rat species. Plourde et al. [18] reviewed human synthesis of long chain polyunsaturates in healthy adults. This group, through an LA tracer supplementation methodology, showed that LA was converted to AA supporting an active Δ5-desaturase in humans. In view of these findings, the second objective of the present study was to determine whether Δ6 can be by-passed by feeding dietary GLA in the presence of an active Δ5-desaturase.

Δ8-Desaturase: An Alternate Pathway? In addition to Δ5-desaturase in the feline species, the possibility of an active Δ8-desaturase exists suggesting a possible alternate pathway for AA synthesis when cats are fed diets high in LA. Although activity has not been specifically established, 1 component of the present study will further address this possibility. Hassam et al. [19] suggested that there was no evidence of Δ8-desaturase in feline EFA metabolism because there was no significant conversion of ^{14}C radio-labeled LA to AA. If the Δ8-desaturase enzyme were present in a specific tissue, AA synthesis from LA could possibly take place through the conversion of 20:2n-

6 to DGLA following Δ8-desaturation demonstrating an alternative pathway for that tissue [9,12]. While some evidence of this pathway exists in other mammals there has been little convincing evidence to date of such an activity. Previous evidence in rat testis, human bladder, and colon demonstrated conversion of 20:2n-6 to DGLA via Δ8-desaturase [10,12,20]. Because cats do show some evidence of Δ5-desaturase activity, if a Δ8-desaturase were active, the cat might first chain elongate LA to 20:2n-6, Δ8-desaturate the product to DGLA, then produce AA via Δ5-desaturase [12]. It should be noted that Park et al. [21] recently demonstrated that when the plasmid containing the coding region from baboon liver for FA desaturase was transformed into S. *cerevisiase*, a yeast with no native biosynthetic ability to synthesize PUFAs, Δ8-desaturase activity was expressed. These findings suggest that baboons possess an alternative pathway for AA synthesis as well as the possibility of an existing Δ8-desaturation pathway in other mammals which may include cats. Thus, the final objective of the study was to determine whether cats can produce 20:3n-6(Δ5,11,14) from dietary LA and whether accumulation of Δ8-desaturase, mainly AA, would occur under the conditions of our experiment.

Summary of Study Objectives. While it is apparent that cats have limited Δ6-desaturase activity, it is unknown whether feeding increased amounts of dietary LA might induce the Δ6-desaturase enzyme to produce either GLA in feline reproductive and adipose tissues or increase the accumulation of 20:2n-6 through chain elongation. Furthermore it is unknown whether bypassing the Δ6-desaturation step with increased amounts of dietary GLA may lead to further chain elongation of the substrate to DGLA, with subsequent production of AA through an active Δ5-desaturase step in feline uterine

tissue. Should the Δ5-desaturase enzyme be active in feline uterine or other tissues, diets containing GLA from plant sources would support the possibility of a vegetarian source of an AA precursor for this species. If so, this novel discovery, through proper nutritional diet formulation, might be a first step toward addressing the expressed need for vegetarian pet owners to have an alternative to the traditional carnivorous diet for their domestic cats. To date, there have been few studies investigating the FA products of Δ5 and Δ8-desaturase activities in cats and no studies using reproductive tissues. Therefore, the objective of this study was to investigate these additional possibilities.

CHAPTER II

MATERIALS AND METHODS

Animals and Diets. Twenty-six, sexually intact female cats ranging between 1½ - 2 years of age were used in this experiment. In accordance with the American Physiological Society Guidelines for Animal Research, all cats were individually kenneled and maintained throughout the study. The cats were cared for by the Laboratory Animal Research and Resources (LARR) staff and resident veterinarian. Members of the Companion Animal Nutrition lab in the College of Veterinary Medicine and Biomedical Sciences monitored and fed the cats daily. These protocols were approved by Texas A&M University Animal Care and Use Committee. Physical examinations, complete blood counts, serum biochemistry profiles, and T_3, thyroid stimulating hormone (TSH) assays were conducted on each cat deeming them clinically normal prior to the study. The cats were then randomly assigned into 1 of 3 groups based on the diet fed. The sample number of cats per group initially started out as 10 per group but 3 were removed from the study as they acquired the Calisie Virus and had to further be euthanized. The fourth cat was removed from the study as she developed a pyometra in which an ovariohysterectomy procedure had to be performed, therefore enabling us to utilize her reproductive tissues. The 3 diet groups to which the remaining cats were randomly assigned into were as follows: High Linoleic Acid (HL, n=7), Low Linoleic Acid (LL, n=9), and ɣ-Linolenic Acid (GLA, n=10). The HL diet consisted of LA-rich safflower oil and, for the LL diet, the safflower oil was replaced with coconut oil in order to reduce LA

to low but adequate levels. The GLA diet was enriched with a high GLA borage oil containing 70% GLA plus an adequate amount of LA. The 3 diets were comparable in all respects excluding FA type (Appendix A, Table A-1). The expected nutrient composition of the diet was: 35% protein, 18% fat, 8% moisture, 7.5% ash, and 2% crude fiber on a dry matter basis. Nestlé Purina Laboratories analyzed the diets after their manufacture and confirmed results that were within the expected analytical variance for the targets (Appendix A, Table A-2). The cats were fed the designated diets for 300 days.

Sample Collection. Blood samples of 7 mL were collected via saphenous veni puncture into EDTA-containing tubes on d0, d140, and d300. Five to six cats were bled per day at approximately 9:00 am after withholding food for 12 hours. Plasma was separated via slow speed centrifugation and used to determine total PL FA composition and total protein concentrations. On d300, food was again withheld for 12 hours and ovariohysterectomies were performed. Five to six cats were spayed per day according to established veterinary practice, and were monitored by LARR's veterinarians and staff after surgery until full recovery. Uterine, ovarian, and subcutaneous adipose tissue samples were collected immediately following ovariohysterectomy procedures. These procedures were completed at Texas A&M University in the LARR facility. Tissues, upon collection, were stored in 9% sodium chloride (NaCl saline solution) on ice during transport to the laboratory then utilized immediately.

Tissue Homogenate Preparations. The tissues, collected on d300, were immediately weighed (g) prior to homogenization. A phosphate buffer was made consisting of 2.139 g of sodium dihydrogen phosphate (NaH_2PO_4) and 6.566 g sodium

phosphate (Na_2HPO_4) per 1 L of distilled water (pH=7.4). An approximate amount of phosphate buffer was added depending on the tissue utilized, Uterine tissue – 1:1 (mL buffer/g tissue), Ovarian tissue – 2:1 (mL buffer/g tissue), Adipose tissue – 2.5:1 (mL buffer/g tissue). The tissues and buffer were combined in a 50 mL plastic Corning® screw top tube where they were thoroughly mixed using a Polytron mixer (Fisher Scientific Tissuemiser) until completely homogenized. The samples were then stored at -80°C until further use.

Preparation of Uterine Tissue Microsomes. Uterine tissues from separate groups of cats that had not been fed defined diets were also collected on 3 separate occasions for microsome preparation throughout the study courtesy of Dr. J. Westhusin in the Reproductive Physiology lab at Texas A&M University. These tissues were utilized for the determination of Δ5-desaturase activity using a ^{14}C radio-labeled DGLA tracer methodology. Adult rat liver tissue was kindly donated by Dr. Gresham from LARR at Texas A&M University for use as a positive control in this experiment because this tissue and species is known to express active Δ5-desaturase activity [16]. Both uterine and liver microsomes were prepared according to Brenner et al. [16]. Tissue preparations were washed in a 9% NaCl solution to clear any additional remnants of blood or connective tissue. Prior to tissue collection, a 40 mM phosphate buffer was prepared using 22.33 g ethylenedramine tetraacetic acid disodium salt (EDTA), 10.89 g potassium phosphate monobasic (KH_2PO_4), 7.46 g potassium chloride (KCl), and 68.46 g sucrose in 1700 mL distilled water. The pH was adjusted to 7.4 with potassium hydroxide (KOH) pellets. Tissues were minced using a razor blade in a petri dish encased in ice, rinsed again with

saline, and finally mixed with the appropriate amount of 40 mM phosphate buffer. The tissues were homogenized in this buffer using the Polytron mixer. Phosphate buffer was added at a 1:1 (w/v) ratio of tissue to buffer. The tissues were homogenized under an ice bath for 3-4 minutes with 30 second breaks allowing the Polytron to cool. This process was carried out in plastic 50 mL Corning® tubes. The tissue homogenates were then transferred to 50 mL (29 x 104 mm) Beckman tubes where centrifugation was performed using an Eppendorf 5810R centrifuge with a F40-6-38 fixed angle rotor equipped with 50 mL tube inserts at 4°C for 20 minutes at 10,000 x g (10,918 rpm). In order to pellet the microsomes, the supernatants were removed and transferred to clean 12 mL Beckman Ultra-Clear centrifuge tubes where they were again centrifuged in a Sorvall Ultra Pro 80 and T-1270 fixed angle rotor for 1 hour at 105,000 x g (33,507.5 rpm). Prior to this centrifugation, the rotor was refrigerated at 4°C in a Du Pont Ultracentrifuge (Du Pont Company, Newtown, CT). After centrifugation, supernatants were aspirated and discarded while the pelleted microsomes were resuspended in 1-2 mL of cold homogenization buffer, (Uterine tissue – 2 mL, Adipose/Ovarian tissue – 1 mL). To ensure comparable consistency between tubes, the volumes were pooled. Protein concentration was determined, and the microsomes were then divided into 0.5 mL aliquots which were stored at -80°C.

Protein Concentration. Protein assays were completed on all feline uterine and rat liver microsome preparations using the Bininchoninic Acid (BCA) Protein Assay kit from Pierce Chemical Co. A 2 mg/ml bovine serum albumin (BSA) standard was prepared by mixing 0.02 g of the BSA standard with 10 mL distilled water and

sequentially diluted to provide an adequate range for detection. Microsome samples were thawed, then diluted if necessary, to fit within the range of protein standards. Microsomes and standards were incubated for 30 minutes at 37°C followed by colorimetric measurement of protein at 550 nm. A UV max® UV/Vis spectrophotometer (Molecular devices, Menlo Park, CA) was utilized for protein concentration measurement.

Lipid Extraction. Fatty acid methyl esters (FAMEs) were prepared by a modified Folch et al. [22] method as described below. The uterine, ovarian, and adipose tissue homogenates along with plasma samples collected at d0, d140, and d300 were all utilized in preparing FAMEs for analysis. Five hundred µL of each sample were transferred into 12 mL Teflon-lined screw topped tubes along with 9 mL of chloroform:methanol (2:1, v/v) containing 0.2% glacial acetic acid. The samples were shaken for 20 minutes on the Shaker-in-the-Round (Kraft Apparatus, Inc., Terre Haute, IN) then 2 mL distilled water was added. The samples were shaken again for 10 minutes then centrifuged at 2800 rpm for 15 minutes at 4°C in the Allegra® X-15R centrifuge (Beckman Coulter). Centrifugation separated the lipid fractions into 2 layers and the subnatants were transferred into clean 12 mL screw top test tubes. Five mL chloroform:methanol:water (3:48:47, v/v/v) was added and the samples were again shaken for 10 minutes. Another 15 minute centrifugation was performed, as previously noted, separating the lipid containing layer into the bottom phase where it was again removed and transferred to the clean 12 mL screw top test tubes containing the initial lipid extract. In order to prevent oxidation, nitrogen gas was streamed over each sample before overnight storage at -20°C.

Thin Layer Chromatography. Thin layer chromatography (TLC) was performed on the lipid extracts to fractionate the lipid samples into major classes. The samples were evaporated to dryness under nitrogen gas and reconstituted with a known amount of chloroform depending on the tissue used (i.e. Uterine – 100 µL, Ovarian – 150 µL, Adipose – 300 µL). Silica gel G TLC plates (20x20 cm Analtech, 15 Newark, DE) were washed in chloroform:methanol (2:1, v/v) containing 0.2% acetic acid. The washed plates were dried overnight in a glassware dryer at 55°C. A TLC chamber containing hexane:diethylether:glacial acetic acid (80:20:1, v/v/v) was lined with chromatography paper (Whatman®) and equilibrated for 1 hour prior to plate development. The washed, heat activated plates were loaded with known amounts of lipid extract depending on the tissue being utilized (Uterine – 25 µL, Ovarian – 75 µL). TLC was not performed on the adipose tissues samples as previous experience has found that the majority of their composition is triglycerides. Three samples were added to each plate in a continuous narrow band along with 20 µL of an #18-5A (Nu-Check Prep, Inc., Elysian, MN) standard in a separate lane for lipid subfraction identification. All plates were developed until the solvent front reached 1 cm from the top of the plate then allowed time to completely dry in a nitrogen flow box (Scientific Manufacturing Industries). The PL fractions, located at the origin of the plate, were scraped into small, clean screw top test tubes and 2 mL of 4% sulfuric acid in methanol were added. Samples were again blanketed with nitrogen and either stored at -20 °C or immediately trans-methylated for analysis.

Trans-Methylation. The resulting samples containing 4% sulfuric acid in methanol were mixed using a Vortex-Genie mixer (Model K-550-G, Scientific Industries Inc., Bohemia, NY) then incubated in a water bath for 1 hour at 90°C. The samples were checked periodically throughout the incubation to assure screw top caps remained tightly sealed. The samples were then cooled to room temperature followed by the addition of 3 mL of hexane. Samples were mixed again, then centrifuged at 2800 rpm for 15 minutes at 4°C. Centrifugation separated the FAMEs into the supernatant layer, which was removed and transferred into a clean screw top test tube. These FAME samples were stored under nitrogen gas at -20°C until further analysis.

Gas Chromatography. The uterine and ovarian tissue FAMEs were evaporated to dryness under nitrogen gas, then reconstituted with 40 µL hexane, 20 µL of which were inserted into a gas chromatography (GC) vial. Adipose tissue FAMEs were prepared under the same conditions but reconstituted with 500 µL hexane instead of 40 µL. The samples were placed on a Hewlett Packard 6890 Series Autosampler. Three µL of uterine and ovarian, and 2 µL of adipose tissue samples were injected onto a FAMEWAX™ (Restek, Bellefonte, PA) fused silica capillary column (30 m long, 0.25 µm thickness, and 0.32 mm ID) in a Hewlett Packard 5890 Series II Gas Chromatograph (Hewlett Packard Co., Palo Alto, CA) with a flame ionization detector. At an initial velocity of 28.3 cm/sec and flow of 1.25 mL/min, helium was the carrier. For 10 minutes the oven temperature was initially held at 170°C, then increased at a rate of 2.0°C per minute until reaching 220°C and held constant for 10 minutes. The temperature was then increased 10°C per minute until a final temperature of 250°C was attained. The

results were generated using a Hewlett Packard ChemStation software package and authentic FAME standards (#68-A, plus 17:0, 18:1n-7, 20:5n-3, and 22:4n-6, Nu-Check Prep, Inc., Elysian, MN) were utilized, based on retention time, to identify the individual FAs.

High Performance Thin Layer Chromatography. The uterine tissue lipid extracts were analyzed for PL distribution using high performance thin layer chromatography (HPTLC), according to Yu et al. [23]. Plastic 20x20cm Silica gel 60 TLC sheets were evenly cut into 10x10cm sheets and heat activated for 30 minutes in a Stabil-Therm Gravity oven (Blue M) at 110°C. The plates were not prewashed as this proved a negative effect on plate background when subsequent charring was performed for visualization. Each individual 10x10 cm plate was loaded with 5 µL of sample from only 1 cat as each plate was to be developed in 2-dimensions. To serve as the first dimensional run, a filter paper lined glass TLC tank was equilibrated with choloform:methanol:water (75:25:2.5 v/v/v) for 1 hour. A second filter paper lined TLC tank was equilibrated for 1 hour using cholorform:methanol:acetic acid:water (80:9:12:2 v/v/v/v) as solvent. The plates were individually developed in the first solvent until the solvent front reached 1 cm from the top of the plate. The plates were then transferred to an enclosed tank where they were dried under nitrogen gas flow for 10 minutes. After allowing sufficient drying time, the plates were developed at right angles to the first development in the second solvent, 1 cm from the top, and then dried again in the nitrogen tank. For visualization, the plates were dipped in a 10% $CuSO_4$ + 80% H_3PO_4 solution, then charred on a Thermoline Type 1900 Hot Plate (Analtech, Deerfield,

IL) set at 180°C under vacuum until prominent spots appeared. The plates were scanned on a Model GS-700 Imaging Densitometer (Bio-Rad) providing a volume analysis report indicating the area (mm^2) of each visualized lipid component which allowed calculation of relative percentage distribution. Authentic standards were chromatographed similarly in order to help identify the PL subfractions.

Phospholipid Phosphorous Assay. The uterine tissue lipid extracts from each of the 26 cats were utilized in this assay according to the Fiske-Subbarow method of Bartlett et al. [24] with some modifications. This is a very sensitive assay therefore all glassware was washed extensively with phosphate-free detergent and dried overnight in a glassware dryer. The standard was prepared by dissolving 0.44 g of dried potassium dihydrogen phosphate (KH_2PO_4) in 100 mL distilled water (1µg/µL). Varying amounts of standard, ranging from 0-40 µL (0, 2, 5, 10, 20, and 40) were carefully pipetted into 12 mL Pyrex centrifuge tubes followed by the addition of 0.25 mL concentrated sulfuric acid (H_2SO_4). The individual feline lipid extracts were evaporated to dryness under nitrogen gas, resuspended in 100 µL chloroform, and then 30 µL of sample was carefully pipetted into 12 mL Pyrex test tubes. The 30 µL samples were evaporated again under nitrogen gas followed by the addition of 0.25 mL concentrated sulfuric acid. All samples were heated in a sand bath for 3 hours at 250°C. While incubating, the Fiske Subbarrow Reagent was freshly made containing 0.05 g purified 1-amino-2-napthol-4-sulfonic acid, 0.1 g anhydrous sodium sulfite, and 20 mL of 15% sodium bisulfate. This reagent was mixed thoroughly then filtered into a brown bottle until use. After the 3 hour incubation, the samples were cooled to room temperature and 2 drops of 30% hydrogen peroxide were

added. The samples were again incubated for 2 hours at 160°C. Post incubation, the tubes were cooled, then diluted with 4.9 mL distilled water, followed by the addition of 0.6 mL of 2.5% ammonium molybdate and 0.25 mL of the Fiske Subbarrow reagent, then thoroughly mixed. Glass marbles were placed on top covering each test tube as a mini-condenser and then samples were heated in a boiling water bath for 10 minutes. Two-hundred µL of each sample was placed into a 96-well microplate and read at 750 nm. The phosphorous assay was utilized to determine the amounts of PLs (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), cardiolipin (CL), sphyingomyelin (SPH), and lyso-phosphatidylcholine (LPC)) present in the uterine tissue homogenates. The mean results (µg) of the phosphorous assay were multiplied by 1000, then divided by 30 as this was the sample amount assayed, then multiplied by 25.4 which is the molecular weight of PC divided by the molecular weight of phosphorus. These values were then divided by the protein concentrations of the homogenate (µg/µL) and results presented as µg PL fraction per mg uterine protein.

DGLA Incubations for Δ5-Desaturase Assay. Uterine microsomal preparations were used to determine Δ5-desaturase activity by measuring percent DGLA conversion to AA. The incubations were performed using fixed microsomal protein concentration, time, and substrate concentration after Brenner et al. [16]. The Δ5-desaturase assay was conducted using a stock solution containing 40 µL of ^{14}C radio-labeled DGLA (ARC Inc. 0381, St. Louis, MO ($3.2x10^6$ cpm/µmol) and 5 µL of DGLA (1.72µmol), evaporated to dryness under nitrogen. It was then resuspended in 200 µL of ethanol, and stored at -20°C. Ten µL of this stock solution was pipetted into individual tubes for the rat liver

incubation preparations (positive control) and 15 µL was used for uterine preparations. For the assays, a 1.5 mL reaction volume was used which contained N-acetyl-L-cysteine (0.00245 g), sodium fluoride (0.0179 g), coenzyme A (0.0004915 g), NADH (0.00615 g), ATP (0.007946 g), magnesium chloride (0.0108 g), sucrose (0.917 g), and potassium chloride (0.12 g) combined in 10 mL of 40 mM potassium phosphate buffer, pH 7.4. For the liver microsomal incubations, the substrate concentration in the final assay mixture was 60 µM with 1.9 mg of microsomal protein added per assay tube. In the case of the uterine preparations, a final concentration of 56 µM DGLA substrate was used in combination with protein amounts of 1.4, 1.8, and 2.6 mg per assay for each of the 3 preparations. Prior to adding the microsomal protein, tubes were pre-incubated for 5 minutes at 37°C with the screw caps removed in a thermoregulated Dubnoff Metabolic Shaking water bath. The reaction was started by adding the specified amount (see above) of liver or uterine microsomes to the respective tubes with subsequent incubation for 30 minutes at 37°C.

Lipid Extraction/Methylation. The reactions were terminated using the Folch et al. [22] lipid extraction solvent previously described. After extraction, samples were stored at -20°C or immediately prepared for methylation. The liver and uterine microsomal lipid extracts were evaporated to dryness under nitrogen gas, followed by the addition of 2 mL 4% sulfuric acid in methanol and methylated. Samples were either stored in -20°C or immediately used for argentation thin layer chromatography.

Argentation Thin Layer Chromatography. Silver nitrate thin layer chromatography was utilized to efficiently separate the ^{14}C radio-labeled DGLA substrate from ^{14}C radio-labeled AA product. Silica gel 60 TLC plastic sheet (20 x 20 cm, EMD Chemicals, Inc.) plates were soaked in 3.5 g silver nitrate in 700 mL methanol for at least 10 minutes, then dried in the Airflow Supreme Fume Hood before being stored in a light protected box because the silver nitrate plates are very light sensitive. The previously methylated liver and uterine samples were evaporated with nitrogen, and reconstituted with 100 µL chloroform. The plates were loaded with 5 µL of each individual liver and/or uterine microsome methyl ester reaction product extracts. A combined standard was constructed including 50 µL of DGLA (5µg/µL) and 50 µL of AA (5µg/µL) (Nu-Chek Prep, Inc., Elysian, MN). Five µL of this standard was placed on top of each sample origin which allowed for subsequent visualization of the FA lipid fractions of interest. The best separation results were established by running 2 separate 1-dimensional runs in which the first glass TLC tank contained hexane:diethylether (90:10 v/v) and the second contained hexane:diethylether (30:70 v/v). Each tank was lined with filter paper and equilibrated for 1 hour. The plates were removed once the solvent front reached 1 cm from the top, then allowed sufficient time to dry in the fume hood. The same plates were placed in the same direction in the second solvent and again removed as the solvent front reached 1 cm from the top. After development and sufficient drying time, the plates were placed in an iodine vapor chamber for 5-10 minutes followed by spraying with a solution containing 0.2 g 2',7'Dichlorofluorescein (Mallinckrodt Baker Inc., Phillipsburg, NJ) in 100 mL ethanol for visualization of the FAs. DGLA and AA were identified and

separately scraped into clean 12 mL glass test tubes. Three mL of hexane:ether (90:10 v/v) was pipetted into each test tube, then carefully mixed. The supernatant was extracted and transferred into 4 mL polyethylene Pony Vials™ (Packard Instruments) using pasture pipettes, then evaporated to dryness under nitrogen. Three and a half mL of Ultima Gold Scintillation Cocktail (Packard Instruments) was added to each vial in preparation for liquid scintillation counting.

Liquid Scintillation Counting. A Packard 1900 TR Tri-Carb Scintillation Counter was utilized to analyze the radioactivity resulting in average counts per minute (CPM). The radioactive polyethylene vials along with a blank vial containing only scintillation fluid, were placed in a counting rack flagged with the appropriate ^{14}C marker (protocol #12) for detection. The samples were individually counted for 4 minutes and average CPM obtained. The blank value was subtracted from the sample values before calculating the percent rate of conversion. The enzymatic activities were reported as nm AA produced per mg protein per minute.

Statistical Analysis. All data were analyzed using SPSS 16.0 for Windows (SPSS Inc, Chicago, Illinois). To determine if the data was normally distributed, the Shapiro-Wilks test was performed. One-Way ANOVA was utilized when data appeared normal to compare the diets with variables throughout the study, i.e. FAs and PLs. Where significant differences were found, Tukey multiple comparison tests were performed among diet groups. Non-normal data was further analyzed using the Kruskal-Wallis test and p-values < 0.05 was considered statistically significant.

CHAPTER III

RESULTS

Animals and Diets. Over the 300 day feeding period, food intake was monitored daily and body weights and condition scores were recorded weekly. It should be noted that a modest decrease in food intake was observed around d300 of feeding the HL diet; however no loss of body weight or body condition was observed during this time. Normal behavioral and physical activity also remained unchanged. A sample of all 3 diets was sent to Dr. Wendy Anderson at Nestlé Purina after 300 days of feeding for analysis (Appendix B, Tables B-2 – B-4). The HL diet results showed an elevated peroxide value in this diet which is an index of oxidation in comparison to the normal range (Appendix B, Table B-2). It is uncertain as to the exact day the diet started becoming oxidized although it likely occurred sometime between d56 and d140 of feeding as a companion study taking place in the Companion Animal Nutrition Lab evaluated blood samples at d56 demonstrating no alterations. In the present work, blood samples were collected on d0, d140, and d300 for complete blood counts and serum biochemistry profiles (Appendix B, Table B-1). On d140 of all 3 diets, the median total plasma protein concentrations of the cats were below the normal range (Clinical Pathology Lab, TAMU-VMTH). In the HL diet group, total plasma protein values were below ideal range, not only on d140, but d300 as well while the other 2 groups were within range. On d0 and d140 of all 3 diets, the median corpuscular hemoglobin concentrations (MCHC) were higher than normal, but within normal range on d300. Nonetheless, there were no

complications during the ovariohysterectomy procedures and all cats made full and healthy recoveries.

Plasma Phospholipid Fatty Acid Profiles. The values on d0 reflected the acclimation diet fed (Nestlé Purina's Kit'n'Kaboodle™) and were equivalent in each group (Table 1).Analysis of plasma PL FAs by gas chromatography generally reflected the diets fed. The GLA diet resulted in both DGLA and AA accumulation in the PL fraction. Feeding the high LA diet resulted in an increase in 20:2n-6 and its putative Δ5-desaturase product, 20:3n-6(Δ5,11,14).

Plasma PL FA data on d140 revealed statistically significantly higher amounts of 16:1n-7, 18:1n-9, and ALA with the LL diet compared with the HL and GLA diets (Table 2). All 3 diets were statistically different from each other regarding LA content. HL contained the highest amount of LA where GLA contained the lowest amount. In animals fed the GLA diet, GLA and DGLA were significantly higher and 20:3n-6(Δ5,11,14) was significantly lower compared to both LL and HL diets. The HL diet resulted in significantly increased amounts of 20:1 and 20:2n-6 and significantly lower amounts of AA versus the other 2 diets. Finally, the total plasma MUFA on d140 was significantly greater in the LL diet versus the HL and GLA diets and total PUFA statistically differed between the LL and HL diets (Table 2).

Day 300 plasma PL FA data coincided with the tissue findings collected at ovariohysterectomy (Table 3). As a direct effect of the GLA diet, GLA was statistically higher along with DGLA. In the LL diet, 14:0, 16:1n-7, and 18:1n-9 were found to be significantly higher. The HL diet demonstrated a variety of statistically significant results

of interest. Significant decreases of 18:0, and ALA were found with the HL diet whereas 20:0, 20:1, and 20:2n-6 were significantly increased. The FAs, LA, 20:3n-6(Δ5,11,14), and AA were each observed to be statistically different among all 3 diets. Highest values of LA and 20:3n-6(Δ5,11,14) were observed in the HL group with lowest values in the GLA group. However, highest values of AA were noted in the GLA group with lowest values seen in the HL group.

The overall relative percentage of SFAs on d300 was significantly lower in the HL group in comparison to the other diet groups and PUFA was statistically lower in the LL group versus the other 2 groups. Regarding the PUFA:SFA ratio in plasma on d300, there was a statistically significant increase observed in the HL group (Table 3).

Uterine Tissue Total Phospholipid Fatty Acid Profiles. The uterine tissue total PL FA profiles revealed the most abundant FAs with all diets to be 16:0, 18:0, 18:1n-9, LA, and AA (Table 4). Lauric acid (12:0) and oleic acid (18:1n-9) were statistically significantly higher in the LL diet group compared with HL and GLA diets. In the LL and GLA diets, myristic acid (14:0) and palmitoleic acid (16:1n-7) were statistically higher versus the HL diet. The FA 20:3n-6(Δ5,11,14), was statistically different among the 3 diets with highest amounts associated with the HL diet and lowest amounts in the GLA group. Eicosadienoic acid (20:2n-6) was significantly higher with the HL diet and both DGLA and AA were statistically higher with the GLA diet. The relative percentages of SFA, MUFA, and PUFA per diet were not statistically different among uterine fatty acid types among the 3 diets. The uterine PUFA:SFA ratios, (Table 4), were also not different.

Table 1 Plasma Phospholipid Fatty Acids – Day 0 (Relative % ± S.D.)

Fatty Acid	Diet LL	Diet HL	Diet GLA
8:0	N/D	N/D	N/D
10:0	N/D	N/D	N/D
12:0	<0.1	<0.1	<0.1
14:0	0.15±0.02	0.16±0.03	0.27±0.06
14:1	N/D	N/D	N/D
15:0	0.10±0.02	0.09±0.03	0.15±0.02
16:0	13.3±0.42	13.0±0.57	14.3±0.91
16:1n7	0.43±0.03	0.35±0.04	0.39±0.03
17:0	0.68±0.01	0.70±0.08	0.72±0.02
17:1	0.05±0.03	0.06±0.03	0.05±0.04
18:0	28.5±0.85	29.2±0.66	29.0±0.66
18:1n9	11.2±0.31	10.7±0.59	10.8±0.38
18:1n7	2.58±0.15	2.19±0.18	2.34±0.19
18:2n6	25.6±0.64	26.0±0.70	23.8±1.02
18:3n6	0.20±0.07	0.21±0.06	0.26±0.06
18:3n3	0.24±0.07	0.26±0.04	0.23±0.05
20:0	0.88±0.06	0.87±0.08	0.96±0.08
20:1	0.44±0.05	0.40±0.04	0.41±0.02
20:2n6	0.65±0.04	0.66±0.08	0.66±0.05
20:3n6 (5,11,14)	0.72±0.03	0.73±0.08	0.61±0.04
20:3n6 (8,11,14)	1.04±0.04	0.97±0.05	1.43±0.13
20:4n6	6.84±0.26	6.78±0.50	6.63±0.44
20:5n3	0.45±0.03	0.35±0.04	0.37±0.04
22:0	0.57±0.08	0.67±0.04	0.68±0.06
22:1	N/D	N/D	N/D
22:4n6	1.18±0.13	1.12±0.35	1.11±0.20
22:5n3	0.55±0.10	0.64±0.22	0.52±0.12
22:6n3	1.26±0.28	1.31±0.41	1.18±0.43
24:0	0.95±0.17	0.92±0.19	0.88±0.23
24:1	1.19±0.04	1.21±0.04	1.09±0.04
SFA	44.58±2.88	45.68±2.71	46.41±3.55
MUFA	15.88±1.36	14.97±0.96	15.13±1.76
PUFA	38.71±2.17	38.50±3.12	36.86±4.13
PUFA:SFA	0.87±0.10	0.85±0.11	0.80±0.13

Values presented as mean ± S.D.
N/D=Not detected
Letters not in common in a row are significantly different, $p < 0.05$
Sample sizes were LL Diet, n=9; HL Diet, n=7; GLA Diet, n=10
SFA=Saturated Fatty Acids; MUFA=Monounsaturated Fatty Acids;
PUFA=Polyunsaturated Fatty Acids

Table 2 Plasma Phospholipid Fatty Acids Day 140 (Relative % ± S.D.)

Fatty Acid	Diet LL	Diet HL	Diet GLA
8:0	N/D	N/D	N/D
10:0	N/D	N/D	N/D
12:0	0.02±0.04	0.00±0.01	0.02±0.04
14:0	0.50±0.21	0.40±0.37	0.41±0.25
14:1	N/D	N/D	N/D
15:0	0.06±0.05	0.22±0.36	0.08±0.05
16:0	13.17±3.11	12.06±2.79	13.13±3.00
16:1n7	0.66±0.19a	0.38±0.11b	0.46±0.13b
17:0	0.26±0.03	0.31±0.07	0.26±0.10
17:1	N/D	N/D	N/D
18:0	27.15±1.12ab	26.16±1.37a	28.19±1.96b
18:1n9	10.60±0.43a	8.05±0.27b	8.78±1.07bc
18:1n7	2.22±0.33	2.35±0.77	1.89±0.37
18:2n6	28.12±1.01a	35.35±3.24b	22.90±2.07c
18:3n6	0.28±0.10a	0.27±0.13a	0.58±0.22b
18:3n3	0.38±0.07a	0.24±0.05b	0.26±0.84b
20:0	0.86±0.25ab	1.12±0.41a	0.71±0.12b
20:1	0.31±0.11a	0.47±0.17b	0.21±0.10a
20:2n6	0.76±0.17a	1.41±0.42b	0.48±0.12c
20:3n6 (5,11,14)	0.85±0.26a	0.75±0.22a	0.42±0.04b
20:3n6 (8,11,14)	2.35±0.55a	1.43±0.41a	10.22±1.92b
20:4n6	5.14±1.19a	2.90±0.46b	5.79±0.91a
20:5n3	0.14±0.06	0.11±0.21	0.10±0.19
22:0	0.71±0.22	0.73±0.41	0.61±0.12
22:1	0.10±0.10	0.89±0.64	0.11±0.08
22:4n6	1.30±0.63	1.18±0.37	1.27±0.29
22:5n3	0.39±0.17	0.27±0.08	0.35±0.21
22:6n3	0.63±0.27	0.37±0.09	0.50±0.22
24:0	1.23±0.41	1.40±0.48	0.97±0.22
24:1	1.55±0.54ab	1.86±0.61a	1.12±0.32b
SFA	43.95±2.16	42.40±2.62	44.37±3.89
MUFA	15.45±1.03a	13.20±1.37b	12.62±1.09b
PUFA	40.34±2.22a	44.28±3.40b	42.86±3.49ab
PUFA:SFA	0.92±0.10	1.05±0.13	0.98±0.15

Values presented as mean ± S.D.
N/D=Not detected
Letters not in common in a row are significantly different, $p < 0.05$
Sample sizes were LL Diet, n=9; HL Diet, n=7; GLA Diet, n=10
SFA=Saturated Fatty Acids; MUFA=Monounsaturated Fatty Acid; PUFA=Polyunsaturated Fatty Acid

Table 3 Plasma Phospholipid Fatty Acids – Day 300 (Relative % ± S.D.)

Fatty Acid	Diet LL	Diet HL	Diet GLA
8:0	N/D	N/D	N/D
10:0	N/D	N/D	N/D
12:0	0.05±0.08	0.03±0.06	0.05±0.12
14:0	0.73±0.30a	0.31±0.19b	0.53±0.28ab
14:1	0.00±0.00	0.02±0.05	0.00±0.00
15:0	0.13±0.06	0.03±0.06	0.13±0.19
16:0	16.45±1.76	14.24±1.82	14.85±2.57
16:1n7	0.91±0.16b	0.48±0.21a	0.67±0.17a
17:0	0.33±0.05	0.36±0.04	0.32±0.11
17:1	N/D	N/D	N/D
18:0	27.29±1.22a	25.24±1.82b	27.97±1.36a
18:1n9	10.66±0.94a	9.20±0.66b	9.22±1.09b
18:1n7	2.24±0.47	2.55±0.49	2.07±0.44
18:2n6	28.52±1.92a	35.70±1.91b	21.46±2.82c
18:3n6	0.29±0.08a	0.23±0.11a	0.57±0.34b
18:3n3	0.35±0.04a	0.16±0.07b	0.30±0.15a
20:0	0.71±0.22a	1.03±0.23b	0.70±0.10a
20:1	0.28±0.10a	0.67±0.19b	0.27±0.07a
20:2n6	0.57±0.13a	1.64±0.43b	0.49±0.12a
20:3n6 (5,11,14)	0.67±0.13a	0.89±0.16b	0.47±0.13c
20:3n6 (8,11,14)	1.87±0.23a	0.83±0.28a	10.57±4.21b
20:4n6	4.12±0.43a	2.16±0.62b	5.26±0.88c
20:5n3	0.00±0.00	0.00±0.00	0.11±0.36
22:0	0.47±0.16	0.57±0.17	0,45±0.17
22:1	0.02±0.05	0.04±0.07	0.13±0.22
22:4n6	0.96±0.30	0.78±0.23	1.09±0.36
22:5n3	0.28±0.11	0.19±0.16	0.32±0.34
22:6n3	0.36±0.21	0.32±0.17	0.36±0.20
24:0	0.78±0.39	0.73±0.47	0.88±0.46
24:1	0.88±0.45a	1.49±0.57b	1.00±0.58ab
SFA	46.92±2.66a	42.561.66b	45.87±3.06a
MUFA	14.99±1.65	14.45±1.35	13.35±1.75
PUFA	37.99±2.08a	42.89±2.05b	41.00±2.79b
PUFA:SFA	0.81±0.09a	1.01±0.08b	0.90±0.11a

Values presented as mean ± S.D.
N/D = Not detected
Letter not in common in a row are significantly different, $p < 0.05$
Sample sizes were LL Diet, n=8; HL Diet, n=7; GLA Diet, n=10
SFA=Saturated Fatty Acids; MUFA=Monounsaturated Fatty Acids;
PUFA=Polyunsaturated Fatty Acids

Table 4 Uterine Tissue Total Phospholipid Fatty Acid Compositions (Relative % ± S.D.)

Fatty Acid	Diet LL	Diet HL	Diet GLA
8:0	N/D	N/D	N/D
10:0	N/D	N/D	N/D
12:0	0.57±0.84a	0.05±0.09b	0.09±0.16b
14:0	1.55±1.14a	0.50±0.26b	1.34±0.58a
14:1	N/D	N/D	N/D
15:0	1.38±1.02	2.12±0.83	1.45±0.86
16:0	19.49±4.01	19.87±1.37	21.13±2.26
16:1n7	0.58±0.36a	0.25±0.15b	0.34±0.19a
17:0	2.77±2.09	3.41±1.28	4.12±2.37
17:1	N/D	N/D	N/D
18:0	17.15±3.46	18.92±2.10	17.01±1.89
18:1n9	14.01±4.0a	11.14±0.80b	10.48±1.43b
18:1n7	2.71±0.53	2.30±0.16	2.87±0.75
18:2n6	13.9±1.1a	16.8± 0.8b	8.6 ±0.3c
18:3n6	0.09±0.23	0.01±0.03	0.05±0.08
18:3n3	0.02±0.07	0.00±0.00	0.07±0.12
20:0	1.73±0.52	2.20±0.42	1.91±0.39
20:1	0.28±0.18	0.35±0.16	0.17±0.15
20:2n6	0.78±0.24a	1.05±0.17b	0.68±0.11a
20:3n6 (5,11,14)	0.62±0.10a	0.86±0.63b	0.38±0.26c
20:3n6 (8,11,14)	1.52±0.5a	1.20±0.46a	4.04±0.86b
20:4n6	12.1±0.7a	10.3±0.8a	15.8±0.7b
20:5n3	N/D	N/D	N/D
22:0	0.75±0.53	0.87±0.62	0.85±0.47
22:1	0.04±0.08	0.03±0.07	0.04±0.08
22:4n6	2.44±1.84	2.06±2.00	2.90±2.44
22:5n3	1.21±1.75	0.53±1.19	1.38±2.20
22:6n3	0.93±0.81	0.90±0.80	0.95±0.80
24:0	0.24±0.30	0.41±0.64	0.40±0.54
24:1	1.62±0.60	1.90±0.37	1.68±0.34
Unidentified	1.39±1.71	0.87±0.68	0.74±0.72
SFA	45.64±4.50	48.35±1.38	48.31±1.63
MUFA	19.27±4.37	14.47±4.19	19.46±13.64
PUFA	33.70±3.97	34.71±2.00	35.36±3.37
PUFA:SFA	0.75±0.14	0.72±0.06	0.73±0.09

Values presented as mean ± S.D.
N/D=Not detected
Letters not in common in a row are significantly different, $p < 0.05$
Sample sizes were LL Diet, n=9; HL Diet, n=7; GLA Diet, n=10
SFA=Saturated Fatty Acids; MUFA=Monounsaturated Fatty Acids;
PUFA=Polyunsaturated Fatty Acids

Table 5 Uterine Microsomal Phospholipid Fatty Acid Profiles of Individual Preparations (Area %)

Fatty Acid	#1	#2	#3
8:0	N/D	N/D	N/D
10:0	N/D	N/D	N/D
12:0	N/D	N/D	N/D
14:0	0.209	0.321	0.317
14:1	N/D	N/D	N/D
15:0	0.083	0.310	0.275
16:0	12.977	22.992	26.603
16:1n7	0.930	0.473	0.396
17:0	0.503	0.876	1.125
17:1	N/D	N/D	N/D
18:0	29.876	17.957	20.522
18:1n9	6.100	9.083	8.609
18:1n7	3.321	3.389	5.267
18:2n6	12.210	10.462	6.165
18:3n6	0.203	0.097	0.293
18:3n3	0.265	0.254	0.212
20:0	0.379	2.203	2.324
20:1	0.200	0.536	0.657
20:2n6	0.460	1.254	0.959
20:3n6 (5,11,14)	0.102	0.560	0.314
20:3n6 (8,11,14)	0.810	1.449	1.068
20:4n6	21.177	13.890	10.000
20:5n3	0.372	0.291	0.000
22:0	0.386	1.326	1.597
22:1	0.000	0.148	0.000
22:4n6	1.702	4.825	4.677
22:5n3	2.771	0.318	1.852
22:6n3	4.088	1.668	1.205
24:0	0.466	1.348	1.885
24:1	0.539	1.994	1.960
Unidentified	0.871	0.517	1.720

N/D=Not Detected

Letters not in common in a row are significantly different, $p < 0.05$

Numbered columns 1-3 signify preparations 1-3

Total uterine microsomal PL FA profiles were determined using each of the 3 pooled tissue microsomes (Table 5). The third preparation of microsomes showed the highest amounts of SFAs and GLA in comparison to the other 2 preparations. Also, both LA and AA were decreased and intermediate amounts of DGLA were observed in the third preparation versus the other 2 preparations.

Ovarian Tissue Total Phospholipid Fatty Acid Profiles. Ovarian tissue total PL FAs were analyzed and calculated utilizing the same procedures as the uterine and plasma samples (Table 6). However, findings revealed very few significant differences among diets. The HL diet was significantly higher in 20:2n-6 versus the other 2 diets. When the GLA diet was fed, statistically higher DGLA compared to the LL and HL diets was found. With regard to total SFA, MUFA, PUFA and the PUFA:SFA ratio, no significant differences were seen (Table 6). Total microsomal PL FA profiles were determined using each of the 3 pooled tissue sources for ovarian tissue as well. These results demonstrated higher amounts of 16:0, 18:0, LA and AA (Table7).

Table 6 Ovarian Tissue Total Phospholipid Fatty Acid Compositions (Relative % ± S.D.)

Fatty Acid	Diet LL	Diet HL	Diet GLA
8:0	N/D	N/D	N/D
10:0	N/D	N/D	N/D
12:0	0.12±0.26	0.38±0.70	0.17±0.35
14:0	1.31±0.36	1.01±0.99	0.95±0.30
14:1	0.09±0.18	0.21±0.27	0.05±0.11
15:0	1.50±1.14	1.03±0.85	1.61±1.02
16:0	24.92±4.42	24.55±6.00	24.48±4.17
16:1n7	0.60±0.34	0.63±0.44	0.46±0.15
17:0	2.70±2.00	2.32±1.64	3.87±1.57
17:1	N/D	N/D	N/D
18:0	16.03±4.52	15.30±3.52	14.55±2.35
18:1n9	11.15±4.10	11.61±4.27	9.86±2.46
18:1n7	4.64±4.10	4.13±1.93	4.28±2.81
18:2n6	8.71±2.64	9.56±4.58	7.68±2.31
18:3n6	0.27±0.56	0.37±0.78	0.39±0.64
18:3n3	0.34±0.65	0.52±0.95	0.19±0.41
20:0	1.46±0.73	1.84±0.70	1.33±0.51
20:1	0.36±0.38	0.60±0.43	0.33±0.31
20:2n6	0.80±0.37a	1.28±0.31b	0.78±0.32a
20:3n6 (5,11,14)	0.41±0.28	0.54±0.43	0.23±0.21
20:3n6 (8,11,14)	1.95±1.18a	1.86±1.40a	3.97±1.13b
20:4n6	11.47±3.33	9.98±2.32	12.62±2.98
20:5n3	1.03±2.07	0.74±0.99	0.29±0.43
22:0	0.69±0.64	1.12±1.09	0.76±0.55
22:1	0.66±1.61	1.10±2.00	0.29±0.72
22:4n6	1.58±2.32	1.33±2.00	2.70±2.73
22:5n3	2.19±2.56	2.15±2.52	3.24±3.14
22:6n3	0.97±1.11	1.38±1.53	1.15±0.80
24:0	0.56±0.82	0.43±0.87	0.49±0.81
24:1	1.56±0.59	1.52±1.07	1.49±0.53
SFA	49.28±8.73	48.02±7.47	48.20±5.30
MUFA	19.01±5.60	19.80±6.47	16.76±4.06
PUFA	29.73±8.06	29.82±4.92	33.41±5.79
PUFA:SFA	0.64±0.23	0.65±0.20	0.70±0.17

Values presented as mean ± S.D.
N/D = Not detected
Letters not in common in a row are significantly different, $p < 0.05$
Sample sizes were LL Diet, n=9; HL Diet, n=7; GLA Diet, n=10
SFA=Saturated Fatty Acids; MUFA=Monounsaturated Fatty Acids;
PUFA=Polyunsaturated Fatty Acids

Table 7 Ovarian Microsomal Total Phospholipid Fatty Acid Profiles of Individual Preparations (Area %)

Fatty Acid	#1	#2	#3
8:0	N/D	N/D	N/D
10:0	N/D	N/D	N/D
12:0	N/D	N/D	N/D
14:0	0.381	0.288	0.235
14:1	N/D	N/D	N/D
15:0	0.312	0.250	0.758
16:0	28.120	24.347	23.051
16:1n7	0.385	0.470	0.399
17:0	0.662	0.743	2.491
17:1	N/D	N/D	N/D
18:0	15.540	15.382	14.099
18:1n9	6.336	8.860	9.789
18:1n7	2.712	3.454	3.186
18:2n6	9.122	10.413	10.043
18:3n6	0.000	0.178	0.205
18:3n3	0.169	0.172	0.178
20:0	0.935	1.248	1.600
20:1	0.231	0.925	0.853
20:2n6	0.862	1.805	1.618
20:3n6 (5,11,14)	0.445	0.560	0.574
20:3n6 (8,11,14)	3.386	2.151	1.806
20:4n6	16.332	12.288	14.593
20:5n3	0.000	0.723	0.000
22:0	0.797	1.017	0.973
22:1	0.193	0.000	0.000
22:4n6	4.696	7.687	7.823
22:5n3	0.916	2.086	0.890
22:6n3	0.695	1.871	1.360
24:0	1.889	0.696	0.784
24:1	1.602	1.529	1.716
Unidentified	3.281	0.857	0.981

N/D=Not Detected

Letters not in common in a row are significantly different, $p < 0.05$

Numbered columns 1-3 signify preparations 1-3

Adipose Tissue Total Fatty Acid Profiles. Adipose tissue FA profiles displayed numerous statistically significant differences but changes were unique compared to reproductive tissue PL profiles (Table 8). The HL diet results were most notable due to a dramatic decline in 12:0, 14:0, 16:0, and 16:1n-7 compared to the LL and GLA diet groups. The two FAs statistically higher in the HL group versus the other diets were LA and 20:2n-6. In the GLA diet, AA was statistically higher compared to the HL diet. The PUFA:SFA ratio was statistically higher with the HL diet also demonstrating that the SFAs were significantly lower and PUFAs significantly higher in adipose tissue from cats fed this diet (Table 8). Other significant findings included higher amounts of both GLA and DGLA in the GLA group.

Uterine Tissue Phospholipid Fractions. The uterine PL fractions were determined using the HPTLC method and identified using authentic standards (Figure 1). The relative percents were calculated to determine the percent of each PL (area mm^2) divided by total PL (area mm^2) then individually multiplied by the total µg PL per mg uterine protein determined from the PL phosphorous assay. On a µg PL per mg uterine protein basis there were no statistically significant differences between the 3 diet groups (Table 9). Phosphatidylethanolamine (PE), PC, and SPH were the most abundant PLs found in the uterine tissues among those identified.

Table 8 Adipose Tissue Total Fatty Acid Profiles (Relative %±S.D.)

Fatty Acid	Diet LL	Diet HL	Diet GLA
8:0	N/D	N/D	N/D
10:0	N/D	N/D	N/D
12:0	8.10±2.11a	2.03±1.14b	7.06±2.55a
14:0	9.07±1.39a	3.55±1.13b	8.23±1.89a
14:1	N/D	N/D	N/D
15:0	N/D	N/D	N/D
16:0	20.12±0.91a	16.22±0.61b	20.44±0.94a
16:1n7	3.11±0.49a	2.05±0.22b	2.92±0.37a
17:0	0.22±0.08	0.22±0.09	0.26±0.03
17:1	N/D	N/D	N/D
18:0	7.00±0.53	7.11±0.73	7.20±0.52
18:1n9	32.34±1.84	31.99±2.64	31.97±2.03
18:1n7	2.36±0.25	2.22±0.16	2.37±0.50
18:2n6	15.2±0.5a	32.2±0.9b	16.2±0.7a
18:3n6	0.07±0.18a	0.03±0.05a	0.56±0.22b
18:3n3	0.49±0.19	0.49±0.22	0.56±0.07
20:0	0.21±0.13	0.21±0.10	0.18±0.09
20:1	0.67±0.12	0.68±0.18	0.59±0.14
20:2n6	0.23±0.12a	0.37±0.04b	0.21±0.08a
20:3n6 (5,11,14)	N/D	N/D	N/D
20:3n6 (8,11,14)	0.12±0.08a	0.06±0.08a	0.58±0.21b
20:4n6	0.15±0.03ab	0.07±0.026a	0.20±0.046b
20:5n3	N/D	N/D	N/D
22:0	0.01±0.02	0.03±0.03	0.01±0.02
22:1	0.01±0.01	0.01±0.01	0.01±0.01
22:4n6	0.16±0.11	0.13±0.11	0.11±0.10
22:5n3	N/D	N/D	N/D
22:6n3	0.00±0.00	0.00±0.01	0.01±0.02
24:0	N/D	N/D	N/D
24:1	0.01±0.02	0.00±0.01	0.01±0.02
SFA	44.74±3.25a	29.43±0.95b	43.46±4.17a
MUFA	38.62±2.11	36.97±2.70	38.06±2.27
PUFA	16.41±1.64a	33.35±2.45b	18.45±2.42a
PUFA:SFA	0.37±0.06a	1.13±0.09b	0.43±0.10a

Values presented as mean ± S.D.
N/D=Not detected
Letters not in common in a row are significantly different, $p < 0.05$
Sample sizes were LL Diet, n=9; HL Diet, n=7; GLA Diet, n=10
SFA=Saturated Fatty Acids; MUFA=Monounsaturated Fatty Acids; PUFA=Polyunsaturated Fatty Acids

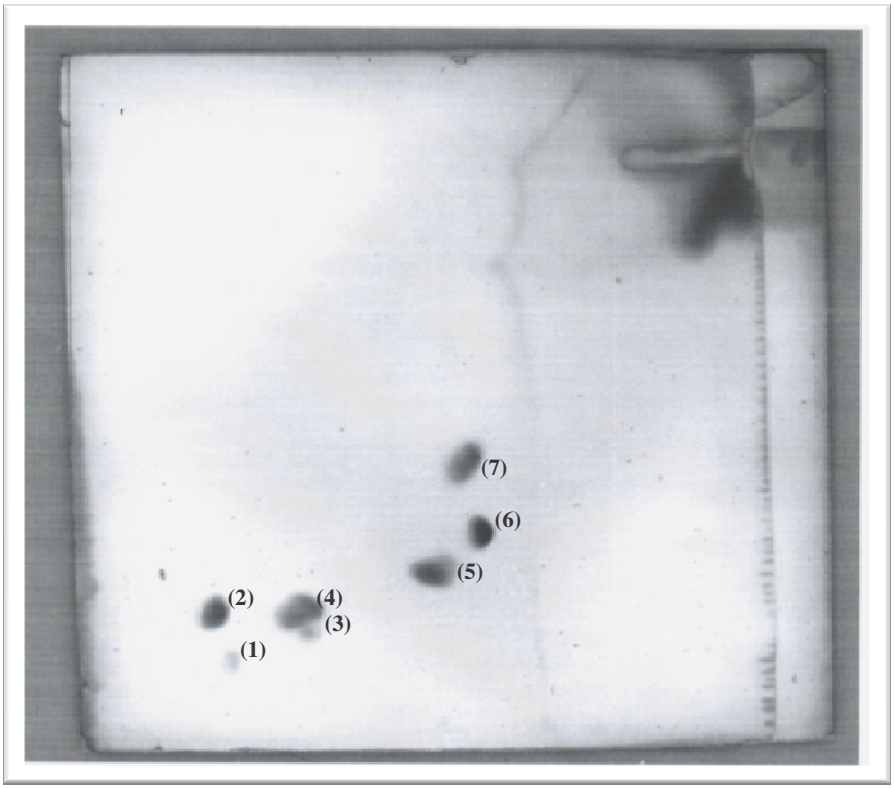

Figure 1 Densitometric Scan of Phospholipid Standards on 2-Dimensional TLC. (1) LPC, (2) PI, (3) SPH, (4) PS, (5) PC, (6) CL, and (7) PE
LPC=Lysophosphatidylcholin; PI=Phosphatidylinositol; SPH=Sphingomyelin; PS=Phosphatidylserine; PC=Phosphatidylcholine; CL=Cardiolipin; PE=Phosphatidylethanolamine

Table 9 Uterine Phospholipid Compositions (µg PL/mg Uterine Protein)

Phospholipid	Diet LL	Diet HL	Diet GLA
PE	28.88±15.38	29.47±12.07	31.56±21.51
PC	24.63±11.21	27.99±7.40	30.13±12.86
PS	10.63±4.08	14.20±5.60	9.29±3.00
CL	3.80±2.30	4.84±5.81	3.00±3.30
LPC	1.62±3.48	0.62±1.64	1.00±2.07
SPH	20.83±8.80	28.36±6.14	26.89±16.50
PI	4.34±4.61	2.42±4.74	1.86±3.16
Unidentified	0.00±0.00	4.71±8.33	1.29±4.08

Values presented as mean ± S.D.
Letters not in common in a row are significantly different, $p < 0.05$
Sample sizes were LL Diet, n=9 HL; Diet n=7; Diet GLA n=10
PE=Phosphatidylethanolamine; PC=Phosphatidylcholine; PS=Phosphatidylserine; CL=Cardiolipin; LCP=Lysophosphatidylcholine; SPH=Sphingomyelin; PI=Phosphatidylinositol

Uterine Microsomal Δ5-Desaturase Activities. Measurement of the Δ5-desaturase activities in the feline uterine tissue microsomes was performed to directly determine the extent of conversion of DGLA to AA as an index of Δ5-desaturase activity. Results are presented as nm AA produced per mg protein per minute.

Using rat liver microsomes the Δ5-desaturase activity was comparable to that of Brenner et al. [16] employing similar methodology with the same tissue. This determination allowed for an acceptable positive control with which to compare the feline uterine microsome activities. Uterine tissue microsomes had been prepared at 3 different times throughout the study using pooled tissues of cats fed various diets. Microsomes were incubated from each of these 3 separate preparations to replicate the Δ5-desaturase activity determinations. Results indicated that feline uterine tissue possesses an active Δ5-desaturase albeit lower than that of rat liver (Table 10). The third uterine preparation yielded a higher enzymatic activity compared to the first 2 preparations. Nevertheless, these results are a significant finding because no studies have been reported to date to directly determine Δ5-desaturase activity in feline uterine tissue.

Table 10 Δ5-Desaturase Enzymatic Activities of Feline Tissue Microsomes

Tissue	nmol/min·mg Protein
Liver (n=3)	1.95±0.40
Uterine (Preparation #1, n=6)	0.20±0.13
Uterine (Preparation #2, n=3)	0.20±0.07
Uterine (Preparation #3, n=4)	0.51±0.13
Uterine Tissue Average	0.30±0.17
Liver – Brenner et al (2002)	1.17±0.20

Values presented as mean ± S.D.

CHAPTER IV

DISCUSSION

Diet Peroxidation Effects. The modest decrease in food intake noted among cats fed the HL group may be linked to the peroxide values in the diet. On d300, the peroxide value was 135.00 meq/kg fat (as-is basis) compared to 17.50 meq/kg fat for the diets at baseline demonstrating that some oxidation had taken place (Appendix B-2). When lipids are exposed to oxygen, a non-enzymatic chemical reaction occurs forming peroxides [25]. The degradation and oxidation of dietary FAs, especially LA, typically presents an unwanted flavor therefore making the food less palatable to the cats. Indeed a modest decrease in food consumption was observed at approximately d300 in the HL diet fed cats although not prior to this time. Because the chain reaction characterizing peroxidation proceeds with time, food consumption may be affected even though in this case it appeared to be only modest. We cannot specifically state whether high peroxide values found in the HL diet had any significant impact on the FA profiles, because the relative percent LA amount remained considerably significantly higher in plasma PL, and tissues in the HL versus the other 2 diets throughout the study as was the HL diet LA content. The LA content of the diet on d0 was roughly 25 relative percent and on d300 (post peroxidation), was 35 relative percent. The relative percent of LA in the plasma PL FAs on d140 and d300 was approximately 35% resulting in no change with time. This data suggests a possible saturation of the PLs with dietary LA which may have taken place within the range of 25-35% dietary LA. If an elevated peroxide value is a hallmark

of oxidized fat then it would be expected that the HL diet sample analysis would have been more dramatically affected which was not the case. A previous study in which peroxidized diets were fed to adult dogs also did not affect plasma FA profiles (unpublished observations). However, because dogs are able to synthesize AA from LA, no drop in AA was noted unlike in the present cat study. This difference may relate to higher AA utilization when cats are fed oxidized diets combined with little synthesis.

The observation of increased peroxidation of the HL diet has raised other questions as to its overall effect on this study. Along with the modest drop in food consumption, a decrease in median total plasma protein concentrations was also observed. A complete understanding of reasons why plasma protein concentration on d140 and d300 were decreased is unknown. Feed intake of cats fed the HL diet was not dramatically decreased nor did they lose body weight and/or condition. Thus, other factors may be involved as these cats were not in a negative energy balance state. One possibility may be due to the typically higher total protein requirement of cats compared to other mammals. As shown in Appendix C, Table C-1, the amino acid profiles of all three experimental diets exceeded both the NRC and AAFCO requirements indicating that adequate protein concentrations were provided [11,26]. Total protein concentrations may have been the result of reduced amino acid availability for protein synthesis, subsequent to protein degradation due to peroxidation. When proteins become oxidized, they are more susceptible to protease attack thereby increasing their rate of degradation [27]. Because cats are in a constant state of gluconeogenesis, one would assume that they would mobilize both protein and fat and lose weight. However, unexplained is the

observation that cats consuming the HL diet maintained their body weights throughout the 300 day period.

Finally, the elevated MCHC on d0 and d140 of cats fed all 3 diets indicates that the hemoglobin concentrations were acceptable but that the red blood cell number was somewhat depressed. This suggests that decreased red blood cell number may be due to depressed protein availability. The decrease in food intake and protein concentrations as well as the elevated MCHC levels were unexpected findings with the HL diet throughout the study, however, the other red blood cell parameters measured stayed within normal limits and the cats remained clinically healthy (Appendix B, Table B-1). It is important to note that these modest changes with the HL diet were not seen in the LL and GLA diets. Thus, results of this study are not believed to have affected the significant findings related to lipid metabolism the latter 2 diets have provided.

Plasma Total Phospholipid Fatty Acids. Plasma samples collected on d0 of the study exhibited no marked changes in total PL FA profiles. These results were expected as all cats had been fed the Nestlé Purina's Kit'n'Kaboodle™ diet for a minimum of 30 days prior to d0 plasma collections.

The plasma PL FA profiles from d140 and d300 closely reflect the experimental diets fed. Statistically higher amounts of 14:0, 16:1n-7 and 18:1n-9 with the LL diet may be the result of higher medium chain triglyceride (MCT) content in which chain elongation and desaturation has occurred. However this effect was not observed when high MCTs plus GLA were fed.

Plasma PL FA results with the GLA diet were similar on both d140 and d300. A statistically significant increase in GLA and DGLA was consistent with findings by Sinclair et al. [12] and Trevizan et al. [28]. When increased amounts of GLA were fed, the essentially absent Δ6-desaturase step appeared to be by-passed in which GLA was chain elongated to DGLA and then further Δ5-desaturated to AA. These plasma PL findings therefore suggest evidence of an active Δ5-desaturase activity in feline species. The GLA diet also showed a statistically significant decrease in 20:3n-6(Δ5,11,14). There was limited to no 20:2n-6 substrate available for Δ5-desaturase to form 20:3n-6(Δ5,11,14) therefore Δ5-desaturase utilizes DGLA for AA synthesis.

The plasma HL group by far demonstrated the most significant changes, some of which may have been due to low concentrations of dietary MCTs and may explain the decreased percent of total saturated fat and 18:0 noted on d300. When dietary MCTs are decreased and animals are in positive energy balance, less chain elongation to 18:0 would be expected. Although MCTs were low in the HL diet, 18:1n-9 was fed in higher amounts compared to the GLA and LL diets and this may explain the statistically significant increase in 20:1 that was detected with the HL diet. Although there have been few studies specifically investigating feline chain elongation, it is anticipated that cats can actively chain elongate FAs explaining this finding.

The significant increases in LA, 20:2n-6 and 20:3n-6(Δ5,11,14) in the HL group on d300 revealed many important aspects that were again consistent with the findings of Sinclair et al. [12] and Trevizan et al. [28]. An increase in LA was expected due to the high amount of dietary LA initially fed with the HL diet and the associated increase of

20:2n-6 demonstrates active chain elongase activity. The increase of 20:3n-6(Δ5,11,14) exhibits a potential alternate pathway of FA metabolism in the cat, as well as the previously noted evidence of active Δ5-desaturation (Figure 2). In the attempt to induce the Δ6-desaturase in cats by feeding high LA, we found that this was not the case given that no accumulation of AA was found in plasma PL of cats fed the HL diet [12]. In fact, this finding confounds our understanding of the AA results in the HL diet. The significant accumulation of AA seen in the GLA diet on d300 was somewhat expected. However reasons for the significant decrease of plasma PL AA in the HL diet fed cats remains unknown. On d140, a statistically significant drop in plasma AA was first observed. This observation may be related to the peroxidation effects noted in the later weeks of the study; the same significant decrease in plasma AA of cats fed the HL diet AA was seen on d300. It is possible that peroxidation effects may have likely depleted cellular AA concentrations resulting in the decreased amounts observed. It should be noted, however, that similar decreases were not observed in other tissues which appeared refractory to this alteration. In order to prove true peroxidation effects on AA metabolism further research needs to be conducted utilizing oxidized diets under carefully controlled conditions.

Another perplexing finding involves the AA accumulation in the LL diet along with the decreased HL-AA displayed on d140 and d300. Dietary AA was equivalent among all 3 diets. We would expect the HL and LL-AA content to be somewhat similar in content to each other with the GLA-AA content increased. With previous evidence showing limited Δ6-desaturase activity in the cat, synthesis of AA from LA is not likely.

However, the possibility exists that 20:3n-6(Δ5,11,14) may be converted to AA through Δ8-desaturase. On d140 we started to see a slight possibility of an active Δ8-desaturase but at d300, this same observation was not perceived. In spite of this latter possibility, little convincing evidence of a functional Δ8-desaturase activity has been shown in mammalian species to this date.

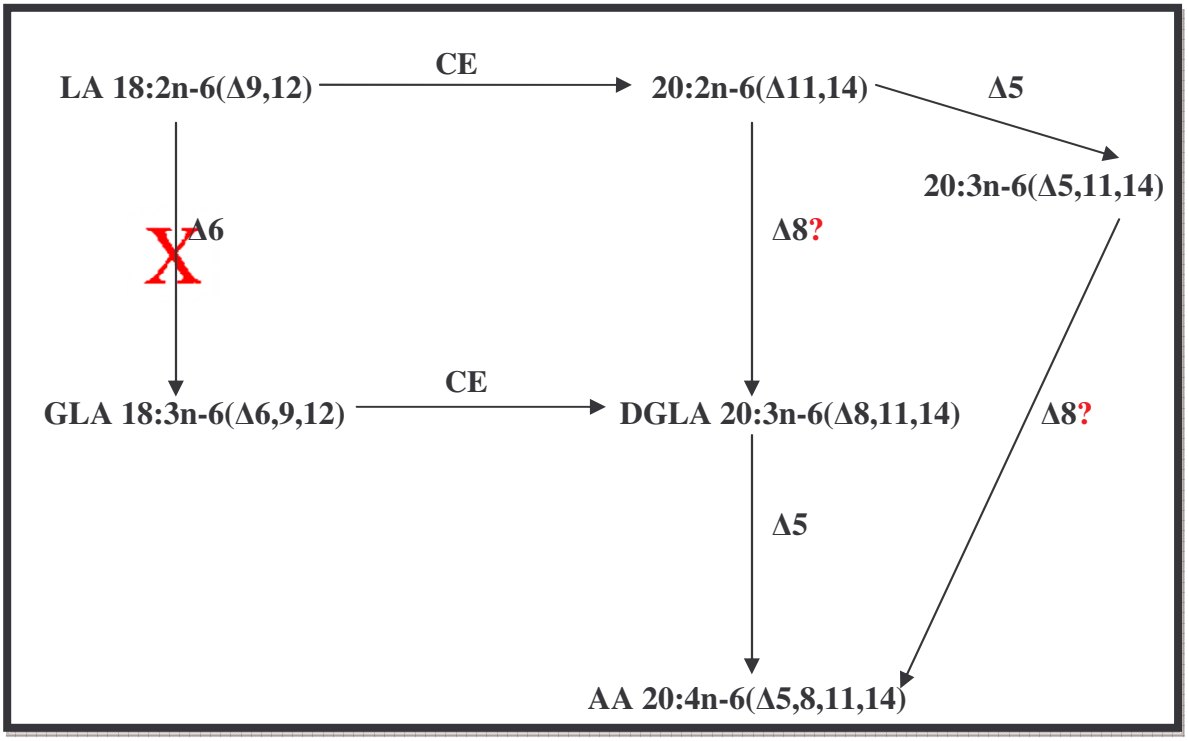

Figure 2 Feline Fatty Acid Metabolism and Structure
CE = Chain Elongation

Uterine Tissue/Microsomal Total Phospholipid Fatty Acids. The uterine tissue PL FA profiles were novel findings in this study. The significant increases of 12:0 and 18:1n-9 in the LL group again, may have been due to the elevated MCTs present in the diet. The increase in both 12:0 and 18:1n-9 suggested enhanced chain elongation of these medium chain FAs, followed by their desaturation.

A fundamental discovery throughout this study was found in uterine tissues of cats fed the GLA diet. Sinclair et al. [14] provided some evidence for Δ5-desaturase activity in feline liver tissue after feeding radio-labeled GLA, by-passing the Δ6-desaturase enzyme step. This work by Sinclair prompted questions as to whether female cats have active Δ5-desaturase activity in reproductive tissues, especially because MacDonald et al. [10] found Δ6-desaturation in testes. In our experiment, we found statistically significant amounts of both DGLA and AA in uterine tissues consistent with by-passing the Δ6-desaturase enzyme step. This finding supports the presence of an active Δ5-deaturase in feline uterine tissues. It suggests the possibility of dietary provision of a functional plant-based form of AA precursor which may ultimately provide an alternative dietary option that may be preferred for vegetarian pet owners. Consequently, through proper diet formulation, borage oil could possibly be utilized as an AA precursor allowing cats to efficiently produce their own conditionally essential AA during times of metabolic demand. Although formulating a nutritionally balanced vegetarian diet for cats requires further exploration, this finding may have introduced a novel future idea for pet food industries.

Another important observation noted in the uterine tissue FA profiles were the higher amounts of AA seen compared to plasma and ovarian tissue AA content. This finding suggests that Δ5-desaturase activity and its subsequent production of AA from GLA may be tissue specific. For instance, if feline liver were actively utilizing Δ5-desaturase for the synthesis of AA from dietary GLA, higher quantities of plasma AA than those seen would have been detected. This result is consistent with findings of Sinclair et al. [12] who concluded that although feline liver cannot synthesize AA from LA and MacDonald et al. [10], who found that feline testes actively can. There are no studies reported to date that have directly determined feline uterine tissue Δ5-desaturase activity. Therefore, the present findings may influence future feline reproductive research.

Although the data in the uterine GLA group were quite dramatic, results from the HL diet were also of interest. Statistically significant increased amounts of 20:2n-6, and 20:3n-6(Δ5,11,14) demonstrate that not only feline plasma actively reflected this pathway, but so does uterine tissue. These findings provide additional evidence for functional Δ5-desaturation occurring in feline lipid metabolism. The synthesis of 20:3n-6(Δ5,11,14) from LA also allows speculation regarding AA production from LA in uterine tissue in the presence of high dietary amounts of LA. For this synthesis to be possible, however, cats would need to possess an active uterine Δ8-desaturase enzyme to convert 20:3n-6(Δ5,11,14) to AA. However, our findings showed statistically lower amounts of AA produced in the presence of high dietary LA suggesting limited or no active Δ8-desaturase activity. Under different conditions, such as EFA deficiency,

Sinclair et al. [14] showed potential evidence of Δ8-desaturase present in cats synthesizing 20:2n-9 from 20:1n-9. Furthermore, Park et al. [21] recently shed new light regarding the detection of Δ8-desaturase in mammals. This group found Δ8-desaturase activity expressed in baboon liver which suggested that this mammal possesses an alternate pathway for AA synthesis. This novel finding advocates the possibility of an existing Δ8-desaturation pathway in other mammals under certain conditions which may include cats.

In addition to the uterine tissue PL FA analysis, we examined the uterine microsomal preparations for the direct measurement of Δ5-desaturation. Activities among the 3 uterine microsomal preparations were somewhat lower but of the same order of magnitude as those of rat liver microsomes [16]. Fatty acid analysis of these feline microsomes demonstrated the lowest amount of AA present in the preparation with the highest activity. Because there was no confirmation available as to the nutritional status, diet, age, etc., the AA content of tissues with lower activity may have caused some feedback inhibition due to its increased presence of AA resulting in lower Δ5-desaturase activity. Another possibility might be that Δ5-desaturase activity could have been induced in some fashion because AA content was the lowest in this preparation with the highest activity thereby increasing demand for its conversion. If so, DGLA would also be present. Intermediate amounts of this FA were detected which is of interest because DGLA is not usually present in typical commercial cat foods. Thus, it may be concluded that the DGLA observed was most likely synthesized from GLA, which was also found to be increased in the third uterine microsomal preparation versus the other 2

preparations. We also noted a decreased amount of LA in the microsome preparation with the higher Δ5-desaturase which may be the result of its use as substrate for chain elongation and Δ5-desaturation. While these conclusions are speculative, further studies in cats may provide additional information on Δ5-desaturation.

Ovarian Tissue/Microsomal Total Phospholipid Fatty Acids. The results obtained while evaluating feline ovarian tissues are somewhat perplexing. Overall, the ovarian FA profiles demonstrated limited differences between diet groups. The statistically significant increase in 20:2n-6 in the HL diet could be due to the high amounts of dietary LA with its subsequent chain elongation. The significant increase in DGLA in the GLA diet group is noteworthy because the lack of AA accumulation in ovarian tissues suggests limited Δ5-desaturase activity in feline ovarian tissues. An alternate explanation, however, may be that any AA accumulation in ovarian tissues for normal cell function could be utilized at rates which equal or exceed AA synthesis.

What is perplexing, however, is that the pooled ovarian microsome total PL FAs demonstrated no distinct marked differences in FA profiles and were similar to the results from the cats fed the defined diets. It is important to note that the data regarding the ovarian microsomes were obtained from cats whose diet history, age, and nutritional status was unknown. Thus, feline ovarian tissue does not seem to be substantially affected by dietary fat modifications. The simple fact that both groups of cats, whether fed a strictly defined diet or not, exhibited similar PL FA profiles, presents an interesting finding regarding metabolism and should be further investigated in the feline species.

Adipose Tissue Fatty Acid Profiles. The adipose tissue FA profiles portrayed considerable differences among the GLA and HL groups but not in the LL group. With the GLA diet, there were statistically higher amounts of GLA present as expected. These elevated amounts of GLA also explain the statistically higher amounts of DGLA present through utilization of an active chain elongase. The accumulation of DGLA in adipose tissue was an interesting discovery. Although AA amounts were statistically different among the HL and GLA groups, this increased AA in the GLA diet was more likely a reflection of plasma in adipose tissues. AA is not known to accumulate in adipose tissue as this tissue contains primarily triacylglycerol and does not typically utilize PL-AA, for eicosanoid synthesis. However, this metabolically active tissue could possibly be a storage reservoir for DGLA which could then be mobilized during times of metabolic stress, trauma or wound healing, etc. In such cases, DGLA would be mobilized and, through Δ5-desaturase, produce AA, and subsequently eicosanoids and prostaglandins. Through cyclooxygenase, the production of these cell mediators would suggest inflammatory or other processes which could instigate an immune or inflammatory response [25].

Not only did the GLA diet group depict differences, the HL group did as well. The significant changes seen in adipose tissue of the HL group may likely be the result of low amounts of MCTs in this diet. As previously mentioned, decreased dietary MCTs would result in more minimal amounts of active chain elongation and desaturation; thus a significant decrease in 12:0, 14:0, 16:0, and 16:1n-7 may reflect this possibility. The PUFA:SFA ratio was also statistically higher demonstrating an increased PUFA and

decreased SFA content and reflects the initial diet composition. The HL diet contained low SFAs and MCTs as well as high amounts of PUFAs in comparison to the other 2 diets. Finally, the statistically significant increase in 20:2n-6 in the HL diet reflected chain elongation of LA. This finding is likely due to the high LA content of the diet and the cats' ability to actively chain elongate but also underscores the lack of Δ6-desaturase activity in the cat.

Uterine Tissue Phospholipid Distribution. There were no statistically significant differences among uterine PLs between diets. The most predominant PLs were PE and PC which is consistent with most tissue PLs. For instance, Norman and Poyser [29] utilized guinea pig uterine tissue for detection of specific prostaglandins. In their experiment, they found both PC and PE to be the 2 main PLs that were acted on during the main mechanism of AA release. It should be also noted that a more profuse amount of SPH was found in feline uterine tissues. This discovery was quite interesting as this FA was not necessarily expected to be predominantly increased. This elevated SPH content, although not statistically significant, may be a unique characteristic of the feline species.

CHAPTER V

SUMMARY

These dietary investigations of feline FA desaturation and elongation have opened a new chapter in understanding feline FA metabolism. While Δ6-desaturase activity has been previously shown to be limited in feline plasma and liver tissues [12], we found comparable results in female reproductive tissues. In our attempt to induce Δ6-desaturase we found evidence of an alternate pathway accumulating both 20:2n-6 and 20:3n-6(Δ5,11,14) in uterine tissue. This suggested that cats do not effectively utilize Δ6-desaturase but may possibly possess an active Δ5-desaturase. This alternate pathway, previously suggested by Sinclair et al. [12] in plasma and liver, has now been shown to be active in uterine tissue as well when higher dietary amounts of LA are consumed.

While Δ6-desaturation activity is limited in the cat, Δ5-desaturase appears to be functional. The likelihood of such activity was noted as part of the previously mentioned alternative pathway, as well as in the second objective of our study in which Δ6-desaturase was by-passed through feeding high dietary amounts of GLA. The presence of the Δ5-desaturase upon feeding increased GLA is evidenced by significant accumulations of both DGLA and AA and suggests the possibility of GLA being utilized as a feed additive precursor for AA in feline uterine tissue. This novel finding could someday benefit pet food industries when looking to target vegetarian pet owners, as GLA could be used as a plant-based source of AA precursor in diets.

Although feline reproductive tissues appear to possess an active Δ5-desaturase, it does not appear that they possess a functional Δ8-desaturase enzyme. If an active Δ8-desaturase were present in uterine tissues, AA would have been synthesized from LA via 2 possible pathways; the synthesis of DGLA from 20:2n-6 or the synthesis of AA from 20:3n-6(Δ5,11,14). Furthermore, based on these findings, it is concluded that feline uterine tissue does not possess a functional Δ8-desaturase when high LA and GLA diets are fed. Overall it can be speculated that cats do in fact show some evidence of Δ5-desaturase activity in uterine tissues, although lack Δ6-desaturase functionality. Further research into feline reproductive FA metabolism, specifically involving desaturase and elongase activities, may be advantageous toward better understanding this species' metabolic activities.

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APPENDIX A

Table A-1 Experimental diet fatty acid profiles[1]

	Diet		
Fatty acids	GLA[2]	HL[3]	LL[4]
	g/kg dry matter		
06:0	0.4	<0.1	0.4
08:0	5.7	2.3	6.0
10:0	4.9	2.3	5.0
12:0	41.2	16.5	43.4
14:0	17.5	7.6	18.4
16:0	29.7	28.9	29.7
16:1(n-7)	4.5	4.5	4.4
18:0	9.3	9.2	9.2
18:1(n-9)	36.8	45.9	36.6
18:1(n-7)	1.8	2.3	1.8
18:2(n-6)	21.8	71.0	20.8
18:3(n-6)	4.2	0.2	<0.1
18:3(n-3)	1.5	2.1	1.4
20:4(n-6)	0.3	0.3	0.3
Saturated	109	68	113
Monounsaturated	45	55	45
Polyunsaturated	28	74	23

[1]Values are the means of duplicate determinations
[2]Borage oil (γ-linolenic acid 70%) plus coconut oil
[3]Safflower oil
[4]Coconut oil

Table A-2 Experimental diet nutrient profile[1]

	Diet		
Nutrient	GLA[2]	HL[3]	LL[4]
	g/kg dry matter		
Crude Protein	346	355	349
Nitrogen-free extract	378	351	378
Fiber	21	18	20
Ash	71	77	70
Fat	185	199	182
Energy, ME kcal/kg[5]	4417	4316	4327

[1]The diets are designated on the basis of their contents. The dry, extruded-type diets were manufactured by Nestlé-Purina Petcare. Diet ingredients (by weight): Brewers milled rice, 35.9%; Soybean protein isolated, 23.3%; Chicken whole carcass and parts, 21.6%; Soybean hulls, 3.67%; Dicalcium phosphate, 2.93%; Coconut oil, 2.80%; Flavor coating, 1.5%; Beef tallow, 0,7%; Potassium chloride, 0.65%; Mineral premix, 0.34%; Choline chloride, 0.32%; Calcium carbonate, 0.29%; Sodium chloride, 0.22%; DL-methionine, 0.18%; Taurine, 0.1%; Vitamin premix, 0.07%; Vitamin E (50%), 0.03%. Vitamin premix contents: 146.32 g/kg nicotinic acid, 10.35 g/kg vitamin A acetate, 90 g/kg dl-α-tocopherol acetate, 84 mg/kg cholecalciferol, 52 g/kg thiamine mononitrate, 51.06 g/kg calcium D-pantothenate, 24.4 g/kg riboflavin, 14.52 g/kg pyridoxine hydrochloride, 6 g/kg folic acid, 508 mg/kg menadione sodium bisulfite, 93 mg/kg vitamin B-12, and 36.8 mg/kg biotin. Mineral mix contents: 65 g/kg zinc as zinc sulfate, 39 g/kg iron as ferrous sulfate, 18.25 g/kg manganese as manganese sulfate, 3.2 g/kg copper as copper sulfate, 651 mg/kg iodine as calcium iodate, and 50 mg/kg selenium as selenium selenite. The remaining percent of each diet were consisted of three dietary oils: Coconut oil, 5.58% in LL diet and Safflower oil, 5.24% in HL diet, which provided the desired fatty acid profiles, Coconut oil 4,85% and Borrage oil at 70% GLA, 0,5%. Values are the means of duplicate determinations

[2]Borrage oil (γ-linoleic acid 70%) plus coconut oil

[3]Safflower oil

[4]Coconut oil

[5]Value obtained by digestibility analyses

APPENDIX B

Table B-1 Complete blood counts of cats during feeding period**.** Values include median values with range in parenthesis

Diet	Days	Total Protein	Albumin	ALP	PCV	MCHC
GLA	0	6.3	3.3	56.6	32.2	35.65
		(5.6-7.1)	(2.8-3.7)	(45-70)	(28.4-37.5)	(34.0-36.6)
	140	5.95	2.8	44.5	32.2	35.85
		(5.2-6.9)	(2.5-3.6)	(41-57)	(28.5-37.0)	(35.4-36.3)
	300	6.15	3.1	29	32	31.55
		(5.5-7.0)	(2.8-3.7)	(23-56)	(27.0-37.0)	(31-34)
HL	0	6.6	3.3	48	36.0	35.9
		(2.6-7.0)	(0.8-3.7)	(25-73)	(30.5-40.0)	(34.3-37.4)
	140	5.3	2.5	54	31.3	36.2
		(4.5-5.7)	(2.0-2.7)	(38-57)	(27.1-34.2)	(35.7-36.8)
	300	5.0	2.8	44	35	32.4
		(4.8-5.7)	(2.5-3.2)	(25-77)	(30-42)	(31.8-33.8)
LL	0	6.3	3.1	45	33.4	36.1
		(5.8-6.7)	(3.0-3.7)	(33-76)	(25.6-38.0)	(35.0-37.0)
	140	5.8	2.8	41	29.8	36.2
		(5.0-6.4)	(2.4-3.0)	(33-51)	(27.1-34.8)	(34.7-37.0)
	300	6.1	3.2	27	36	32.7
		(5.5-6.9)	(2.8-3.4)	(17-42)	(29.2-38.0)	(30.0-34.9)
Normal Range		**6.1-7.7**	**2.5-3.3**	**20-109**	**24-45**	**31-35**

Table B-2 HL Diet Profile

	As Is		Dry Matter		ME (kcal/100g As Is)	
	Baseline[a]	15 Months Later[b]	Baseline	15 Months Later	Baseline	15 Months Later
Moisture (%)	8.25	9.17				
Protein (%)	32.60	30.90	35.53	34.02	114.10	108.15
Fat, acid hydrolysis (%)	18.30	16.80	19.95	18.50	155.55	142.80
Saturated (g/100g)	5.64	4.88				
Monounsaturated (g/100g)	4.33	3.09				
Trans (g/100g)	0.14	0.11				
Polyunsaturated (g/100g)		2.03				
Total fat (g/100g)	16.80	10.90				
Fiber, crude (%)	1.65	1.74	1.80	1.92		
Ash (%)	7.04	6.75	7.67	7.43		
Peroxide (meq/kg fat)	**17.50**	**135.00**				
NFE = (100-(Protein+Fat+Fiber+Ash+Moisture))	32.16	34.64	35.05	38.14	112.56	121.24
				Total	**382.21**	**372.19**

a: Reported in 06/28/2007 by N.P. Analytical laboratory in Nestle Purina PetCare Company
Diet was made 06/2008 by Nestle Purina PetCare Company

b: Reported in 10/06/2008 by N.P. Analytical laboratory in Nestle Purina PetCare Company

Table B-3 LL Diet Profile

	As Is		Dry Matter		ME (kcal/100g As Is)	
	Baseline [a]	15 Months Later [b]	Baseline	15 Months Later	Baseline	15 Months Later
Moisture (%)	8.36	8.46				
Protein (%)	32.00	31.50	34.92	34.41	112.00	110.25
Fat, acid hydrolysis (%)	16.70	16.60	18.22	18.13	141.95	141.10
Saturated (g/100g)	9.05	8.99				
Monounsaturated (g/100g)	3.38	3.32				
Trans (g/100g)	0.12	0.12				
Polyunsaturated (g/100g)		1.54				
Total fat (g/100g)	15.20	14.80				
Fiber, crude (%)	1.87	2.11	2.04	2.31		
Ash (%)	6.45	6.22	7.04	6.79		
Peroxide (meq/kg fat)	6.54	2.12				
NFE = (100-(Protein+Fat+Fiber+Ash+Moisture))	34.62	35.11	37.78	38.35	121.17	122.89
				Total	**375.12**	**374.24**

a: Reported in 06/28/2007 by N.P. Analytical laboratory in Nestle Purina PetCare Company
Diet was made 06/2008 by Nestle Purina PetCare Company
b: Reported in 10/06/2008 by N.P. Analytical laboratory in Nextle Purina PetCare Company

Table B-4 GLA Diet Profile

	As Is		Dry Matter		ME (kcal/100g As Is)	
	Baseline [a]	15 Months Later [b]	Baseline	15 Months Later	Baseline	15 Months Later
Moisture (%)	7.41	7.95				
Protein (%)	32.00	31.90	34.56	34.66	112.00	111.65
Fat, acid hydrolysis (%)	17.10	16.70	18.47	18.14	145.35	141.95
Saturated (g/100g)	8.98	8.52				
Monounsaturated (g/100g)	3.48	3.22				
Trans (g/100g)	0.13	0.11				
Polyunsaturated (g/100g)		1.79				
Total fat (g/100g)	15.70	14.40				
Fiber, crude (%)	1.93	1.87	2.08	2.03		
Ash (%)	6.54	6.22	7.06	6.76		
Peroxide (meq/kg fat)	9.33	5.80				
NFE = (100-(Protein+Fat+Fiber+Ash+Moisture))	35.02	35.36	37.82	38.41	122.57	123.76
				Total	**379.92**	**377.36**

a: Reported in 06/28/2007 by N.P. Analytical laboratory in Nestle Purina PetCare Company
Diet was made 06/2008 by Nestle Purina PetCare Company

b: Reported in 10/06/2008 by N.P. Analytical laboratory in Nestle Purina PetCare Company

APPENDIX C

Table C-1 Amino Acid Comparisons Among Diets and Recommendations

Amino Acid	NRC	AAFCO	LL	HL	GLA	NRC	AAFCO
	g/1000 kcal ME	g/1000 kcal ME	g/100g	g/100g	g/100g	g/100g	g/100g
Aspartic Acid	N/A	N/A	3.39	3.40	3.45	N/A	N/A
Threonine	1.3	1.83	1.15	1.15	1.17	0.52	0.732
Serine	N/A	N/A	1.54	1.52	1.55	N/A	N/A
Glutamic Acid	N/A	N/A	5.78	5.73	5.83	N/A	N/A
Proline	N/A	N/A	1.71	1.72	1.72	N/A	N/A
Glycine	N/A	N/A	1.50	1.49	1.50	N/A	N/A
Alanine	N/A	N/A	1.52	1.50	1.52	N/A	N/A
Valine	1.28	1.55	1.59	1.59	1.60	0.512	0.62
Isoleucine	1.08	1.3	1.34	1.34	1.37	0.432	0.52
Leucine	2.55	3.1	2.54	2.50	2.55	1.02	1.24
Tyrosine	N/A	N/A	1.12	1.09	1.12	N/A	N/A
Phenylalanine	1	1.05	1.55	1.53	1.56	0.4	0.42
Histidine	0.65	0.78	0.74	0.73	0.75	0.26	0.312
Lysine	0.85	2.08	1.90	1.90	1.94	0.34	0.832
Arginine	1.93	2.6	2.31	2.29	2.33	0.772	1.04
Methionine & Cystine	0.43	1.55	0.64	0.62	0.70	N/A	0.011
Tryptophan	0.33	0.4	0.34	0.35	0.36	0.132	0.16
Taurine	0.1	0.25/0.50*	N/A	N/A	N/A	0.04	N/A

N/A=Not Available
AAFCO=Association of American Feed Control Officials
NRC=National Research Council
AAFCO Values taken from Adult Maintenance Minimum
NRC Values taken from Adequate Intake of Adult Cats for Maintenance
The LL, HL, GLA diets are g amino acid/100 grams food
The NRC and AAFCO nutrient profiles presumes an energy density greater than 4000 kcalME/kg
*Taurine for AAFCO 0.25=Extruded; 0.50=Canned

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Publications: **Chamberlin AJ**, Mitsuhashi Y, Bigley KE, Bauer JE (2009) A vegetarian dietary oil source of ɣ-linolenic acid results in arachidonic acid accumulation in feline uterine tissue: Evidence of delta-5 desaturation. Intercollegiate Faculty of Nutrition, Spring Symposium, Texas A&M University

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